Preclinical evaluation of coronary vascular function after cardioplegia with HTK and different antioxidant additives

Cornelia Schröder a, Anke Heintz b, Annette Pexa a, Ursula Rauen c, Andreas Deussen a, *

a Institute of Physiology, Medical Faculty Carl Gustav Carus, TU Dresden, Germany
b Clinic of Anaesthesiology and Intensive Care Therapy, Medical Faculty Carl Gustav Carus, TU Dresden, Germany
c Institute of Physiological Chemistry, University Hospital, Essen, Germany

Abstract

Objective: Due to limited resources, improvement of preservation solutions is still of great importance in cardiac transplant surgery. New additives with antioxidant properties were tested with respect to coronary function of isolated rat hearts. Methods: Bretschneider HTK solution containing none or an antioxidant additive (deferoxamine, trolox or LK 616) was used for 8 h cold cardioplegia. After reperfusion with Krebs-Henseleit buffer (KHB), we assessed vascular dilator capacity (bradykinin, adenosine triphosphate, reactive hyperemia), myocardial function (left ventricular developed pressure, heart rate, oxygen consumption) and release of biochemical markers (aspartate aminotransferase, creatine kinase, lactate dehydrogenase, troponin, adenosine). Results: Bradykinin- and adenosine triphosphate-induced vasodilations were largely reduced in hearts stored 8 h in traditional HTK as compared to unstored controls. Storage in HTK + LK 616 significantly improved bradykinin-induced vasodilation. Vasodilation toward ATP was best preserved in hearts stored in HTK + deferoxamine. Deferoxamine and trolox, both improved reactive hyperaemic response during reperfusion. Left ventricular pressure development was significantly reduced after 8 h cardioplegia, but no difference existed between different cardioplegia groups. Release of biochemical markers of tissue injury was similar in all cardioplegia groups. After storage in HTK + LK 616 (100 \text{ M}), however, heart marker release was slightly augmented as compared to HTK. Conclusions: Despite similar myocardial function and marker release, coronary vascular function after cardioplegic storage may profit by addition of iron chelators (or antioxidants) to traditional HTK solution.

Keywords: Coronary flow; Deferoxamine; Langendorff heart; LK 616; Radical scavengers; Trolox

1. Introduction

The demand for donor hearts for cardiac transplant surgery exceeds the supply (adapted from Eurotransplant: http://www.eurotransplant.nl). This implies that organ preservation should be further optimized in order to maximize the yield of successful transplantations, e.g. by extension of the possible storage time. Cardioplegic solutions have been developed more than two decades ago [1], at that time the role of the endothelium for proper vascular function was not recognized [2]. More recently, it has become evident that a marked injury of endothelial cells occurs during cold storage [3,4]. This implies that in order to prevent cold injury of vascular function, cardioplegic solutions should contain additives that preserve the endothelial cell.

Cold injury of endothelial cells is largely triggered by mechanisms that involve oxidative stress [5]. To minimize cell injury through oxidative stress, it is necessary to either reduce production of oxygen radicals, to scavenge oxygen radicals effectively or to block the conversion of reactive species of low reactivity (e.g. hydrogen peroxide) to species of high reactivity (e.g. hydroxyl radicals). Transition metals, especially iron, are crucially involved in the formation of these highly reactive species, and an increased availability of 'redox-active', chelatable iron has proven to be the decisive factor in cold-induced injury of diverse cells [3,5–7]. In the present study, several additives with antioxidative properties (iron chelators, oxygen radical scavengers) were tested with respect to their effect on vascular function in rat hearts stored for 8 h in traditional HTK solution at 4 °C. The time range was chosen to exceed the usual maximum storage time of human hearts, thus creating stiff testing conditions for this screening study.

2. Materials und methods

Male Wistar rats (n = 50) with a body mass of 200–350 g were purchased from Charles River GmbH (Wiga, Germany).
The animal care and all experimental procedures were performed in accordance with the animal welfare regulations of the German local authorities, which conform to NIH guidelines.

2.1. Heart isolation

Following anaesthesia with urethane (1.5 ml/100 g body mass i.p.), heparin (2500 IU/100 g body mass i.p.) was given before the abdomen was opened transversally, the diaphragm cut and cardioplegic solution (4 °C) instilled into the thorax. Following opening of the thorax, a cannula was inserted retrogradely from the aortic arch toward the heart. The inferior caval vein was opened to prevent overstretching of the right heart. Cardioplegic solution (HTK ± additives, 10 ml, 4 °C) was applied until blood was removed from the coronary vessels and the heart arrested. The heart was excised and stored in the respective cardioplegic solution for 8 h at 4 ± 1 °C. Solutions were freshly prepared on the day of use. The basic cardioplegic solution was HTK (Custodiol®), kindly provided by Dr Franz Köhler Chemie GmbH, Alsbach-Hähnlein, Germany and contained NaCl 15 mmol/l, KCl 9 mmol/l, MgCl₂ 4 mmol/l, histidine hydrochloride 18 mmol/l, histidine 180 mmol/l, tryptophan 2 mmol/l, mannotol 30 mmol/l, CaCl₂ 0.015 mmol/l, and α-ketoglutaric acid monopotassium salt 1.0 mmol/l. The following substances were added to HTK solution: deferoxamine (D, 1 mmol/l; Novartis Pharma, Nuremberg, Germany), trolox (T, 1 mmol/l; kindly provided by Dr Franz Köhler Chemie GmbH), and L-arginine (L, 10 or 100 μmol/l).

2.2. Langendorff heart experiments

Following storage, hearts were reperfused at a pressure of 96 ± 2 mmHg with a modified Krebs-Henseleit buffer (KHB) containing NaCl 116 mmol/l, KCl 4.6 mmol/l, MgSO₄ 1.2 mmol/l, KH₂PO₄ 1.2 mmol/l, NaHCO₃ 25 mmol/l, CaCl₂ 2.5 mmol/l, Glucose 11 mmol/l and pyruvate 2 mmol/l. The buffer was equilibrated with carbogen gas (resulting pH 7.38, O₂ approximately 640 mmHg) and maintained at 37 °C. Coronary venous effluent perfusate was sampled anaerobically with a blood gas analyzer (AVL 990S, AVL Chemistry, University Hospital, TU Dresden). Concentrations of the hypoxia marker adenosine were assessed by HPLC techniques using an etheno-derivatization in conjunction with fluorescence spectroscopy [8].

Coronary vascular function was assessed using the following tests: (1) flow increase after a bolus (0.5 μmol/l) of adenosine triphosphate (ATP), (2) flow response during infusion of bradykinin at an intravascular concentration of 1 nmol/l, and (3) reactive flow overshoot after a 20 s complete flow stop.

2.3. Experimental groups

Hearts (n = 36) were stored for 8 h at 4 °C in the respective cardioplegic solution and then reperfused for 120 min with KHB at 37 °C. The identical solution used for storage of a heart, was used for arrest during the preparation procedure. Control hearts (n = 8) were arrested with cold HTK, but they were reperfused immediately after excision without further storage. These hearts served as an internal control for myocardial and vessel functions without cardioplegic storage. Another three hearts stored for 8 h at 4 °C in KHB (initially oxygenated and buffered to pH 7.4) were used to quantify the extent of injury, if no specific cardioplegic storage precautions are observed. Yet another three hearts were immediately transferred to storage at 37 °C in a sealed container with KHB (initially oxygenated and buffered to pH 7.4) to simulate 1 h of global ischemia. This group served as another model internal control group to facilitate interpretation of the extent of injury as assessed by the release of heart markers in the isolated perfused heart model.

2.4. Calculations and statistics

Myocardial oxygen consumption (VO₂, μL/min) was calculated from the arterial-venous difference of pO₂ according to Fick’s principle with the use of Bunsen’s absorption coefficient (α = 0.0316 μL/mmHg/ml) at 37 °C [9]. Data reported are mean values ± S.E.M. Adenosine concentrations were evaluated using one-way ANOVA. A two-factor ANOVA was applied for all other parameters to test for differences between groups and time-dependent effects. Contrasts were assessed using a post hoc test (LSD). A P-value of 0.05 (two-tailed) was considered to indicate a significant difference. For statistical analyses, SPSS (Version 11.0) was used.

3. Results

In hearts, which were immediately reperfused after isolation, ATP bolus injection (0.5 μmol/l) increased coronary flow by 91 ± 14% and 115 ± 26% after 60 and 120 min reperfusion, respectively (Fig. 1). ATP-induced vasodilation was significantly depressed in hearts stored in HTK as compared to hearts which were immediately reperfused. In hearts stored in HTK + D the flow rise toward ATP after 60 min reperfusion was similar to that seen in immediately reperfused hearts. In all other groups, the vasodilation was severely depressed after 60 min reperfusion, as observed for HTK without additives. With ongoing reperfusion, the flow
rise toward ATP increased in hearts stored in HTK + L (100 μmol/l), HTK + T, HTK and KHB, with values in the groups with the antioxidant additives (HTK + D, HTK + T, HTK + L, 100 μmol/l) being notably higher than in the group without antioxidant additive (HTK).

In hearts immediately reperfused after isolation intracoronary bradykinin (1 nmol/l) increased coronary flow significantly by 28 ± 7% and 28 ± 8% after 60 and 120 min, respectively (Fig. 2). In contrast, in hearts stored in HTK coronary flow response following bradykinin was completely abolished for the entire duration of reperfusion. There even existed a trend toward bradykinin-induced flow reductions after 120 min. Bradykinin-induced flow increases, however, were preserved in hearts stored in the presence of antioxidants, best in hearts stored in HTK + L (100 μmol/l) and partially in hearts stored in HTK + T and HTK + D, respectively.

The reactive flow response following a flow stop of 20 s was 31 ± 4% and 44 ± 5% after 50 and 110 min of reperfusion, respectively, if hearts were immediately reperfused after isolation (Fig. 3). In all groups with cardioplegic storage, the reactive coronary flow overshoot was depressed (<15% of control) after 50 min reperfusion. During the further course of reperfusion, the reactive flow response tended to recover in all groups. However, in hearts stored in traditional HTK, recovery was less than in hearts stored in HTK + D and HTK + T.

In Table 1 pressure development and heart rate data are summarised. In general, developed left ventricular pressure decreased during the time of reperfusion, while heart rate (unpaced) remained constant. Pressure development was systematically lower during reperfusion after 8 h of cardioplegic arrest than in the control group (P < 0.01). No differences in heart rate were observed. There were no significant differences in pressure development or heart rate between the various cardioplegic groups. However, whereas all hearts were spontaneously active when reperfused immediately after isolation, seven out of eight hearts resumed beating after 8 h storage in HTK, HTK + D, and HTK + T, respectively. In hearts stored with HTK and an additive of 100 μmol/l LK 616 only four out of eight hearts resumed beating. In hearts stored with an additive of 10 μmol/l LK 616, four out of four hearts resumed beating; however, it should be noted that vascular function was not improved as compared with storage in KHB without additives.

In hearts immediately reperfused after isolation, release of ASAT, CK, LDH and troponin was at or below the detection limit. Release of biochemical markers of myocardial injury is

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of reperfusion</th>
<th>Heart rate (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Control</td>
<td>103 ± 7</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>HTK</td>
<td>68 ± 10*</td>
<td>57 ± 6*</td>
</tr>
<tr>
<td>HTK + D</td>
<td>57 ± 8</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>HTK + T</td>
<td>76 ± 6*</td>
<td>55 ± 4*</td>
</tr>
<tr>
<td>HTK + L</td>
<td>76 ± 8</td>
<td>54 ± 4</td>
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</table>

Data refer to hearts spontaneously active after reperfusion, which was 8/8 for control, 7/8 for HTK, HTK + D, and HTK + T, respectively, and 4/8 for HTK + L.

Control group: hearts reperfused immediately after isolation without cardioplegic storage. HTK: histidine—tryptophan—ketoglutarate; D: deferoxamine; T: trolox; L: LK 616. Concentrations of additives used were deferoxamine 1 mmol/l, trolox 1 mmol/l, LK 616 100 μmol/l.

P < 0.01 versus control for the respective reperfusion period.

Fig. 1. ATP (0.5 μmol/l) induced coronary flow increase after 60 and 120 min of reperfusion. T: trolox; D: deferoxamine; L: LK 616 (100 μmol/l); HTK: histidine—tryptophan—ketoglutarate. *P < 0.05 versus control.

Fig. 2. Bradykinin (1 nmol/l) induced coronary flow increase after 60 and 120 min of reperfusion. For abbreviations see legend to Fig. 1. *P < 0.05 versus control; #P < 0.05 versus HTK.

Fig. 3. Reactive coronary flow responses following 20 s flow stop after 50 and 110 min of reperfusion. For abbreviations see legend to Fig. 1. *P < 0.0001 versus control.
Coronary venous concentration of adenosine is a sensitive parameter of acute myocardial hypoxia [9]. Following 8 h storage in HTK, coronary venous adenosine concentration (20 ± 3 nmol) during 120 min of reperfusion was similar to that measured in the control group without cardioplegic storage (14 ± 1 nmol). However, adenosine concentration in groups HTK + L (29 ± 4 nmol, LK 616 concentration 100 μmol/l) and HTK + T (26 ± 4 nmol/l) was significantly elevated compared to HTK. Baseline coronary flow, arterial-venous difference of pO₂ and myocardial oxygen consumption were similar in all groups (data not shown).

### 4. Discussion

Cardiac tissue obtained for transplantation is usually stored for a few hours under hypothermic conditions. Storage in Bretschneider HTK solution reduces the energy requirements of the tissue and therefore supports resumption of contractile function following reperfusion (Table 1). However, recent studies on isolated cells have documented the occurrence of a specific cold-induced injury that is mediated by reactive oxygen species formed in an iron-dependent way [5]. In contrast to many other types of free radical-mediated tissue injuries, this injury is not due to an increased cellular release of superoxide anion radicals and/or hydrogen peroxide (the release of these species is even decreased during cold incubation), but is initiated by a cold-induced increase in the intracellular chelatable iron pool [10]. This pool, physiologically only accounting for a minor part (about 1%) of cellular iron, is involved in the conversion of reactive oxygen species of low reactivity (hydrogen peroxide) to reactive oxygen species of high reactivity (hydroxyl radicals and iron-oxygen species) [11]. The cold-induced increase in chelatable, 'redox-active' iron leads to lipid peroxidation, a mitochondrial permeability transition, free radical-mediated cell injury and cold-induced apoptosis in various cell types [3,5,7,11,13], including different types of endothelial cells [3,5].

This iron-dependent cold-induced injury is not addressed by preservation solutions currently in clinical use [14]. As evident from Figs. 1–3 the use of a traditional cardioplegic solution, Bretschneider HTK, also did not offer good endothelial preservation in the whole heart as indicated by three independent indices of coronary regulatory functions (vasodilations toward bradykinin and ATP, reactive hyperaemic response). Reactive hyperaemia is largely mediated by activation of K⁺ATP channels, most likely present on vascular smooth muscle cells, and involvement of the myocardial metabolite adenosine [15]. Bradykinin has been shown to act via stimulation of endothelial NO release [16]. ATP may act via enhanced NO release or by stimulation of P₂ receptors on smooth muscle cells [17]. Flow response to ATP stimulation may also include adenosine signalling (P₁ receptors) [18] evoked by the degradation of ATP via ecto nucleotidases [19]. After 8 h storage of rat heart in traditional HTK solution, we observed a loss of the coronary hyperaemic response, the complete loss of bradykinin-induced coronary flow rise and a partial loss of ATP-induced vasodilation.

To improve post-cardioplegic coronary flow regulation different additives with antioxidative properties were screened in the present study. Deferoxamine forms a complex with Fe³⁺ ions, and thereby decreases formation of hydroxyl radicals or iron-oxygen species. Due to its high molecular weight (molecular mass = 561 g/mol) and hydrophilic nature, however, this compound has limited membrane permeability. It has been used in experimental studies on ischemia and reperfusion and was shown to reduce infarct...
size development after transient acute regional ischemia [20] and to lower reperfusion injury when applied during cardioplegia and reperfusion [21]. LK 616 is a smaller, more lipophilic and thus, more membrane-permeable chelator. It is based, as deferoxamine, on hydroxamic acid as the chelating motif. Trolox is a classical antioxidant, a water-soluble α-tocopherol (vitamin E) derivative. It interacts with peroxyl and alkoxyl radicals, and may thus interrupt oxygen radical chain reactions [22]. Trolox has been shown to protect microvascular function after short duration global ischemia (45 min), if applied during the reperfusion period [23].

As illustrated in Figs. 1–3, hearts stored with HTK augmented with one of the additives showed improved vasodilatory function as compared to traditional HTK. Especially addition of LK 616 (100 μmol/l) resulted in a significantly better response to bradykinin than HTK. This was not the case for the lower concentration of LK 616 tested (10 μmol/l). However, LK 616 in a concentration of 100 μmol/l impaired spontaneous mechanical activity upon reperfusion. The reason was probably a failure of electrical activation or electrical conduction. A slight LK 616 dependent injury was also indicated by the small but significant rise of cardiac marker release (Table 2). These experiments have shown that both iron chelators can protect the vascular endothelium, although the weaker chelator LK 616 only at the higher (but toxic) concentration when employed alone. For future applications, the combination of the weaker, but membrane-permeable LK 616 (in a low concentration, e.g. 10 μmol/l) and the stronger but poorly membrane-permeable deferoxamine is envisaged, with the permeable LK 616 expected to provide a first line of defence but subsequently to transfer the iron ions to the stronger deferoxamine. This approach has shown promising results in an isolated vessel model using 100 μmol/l deferoxamine and 10–20 μmol/l of the LK 616 isomer LK 614 (T. Wille et al., unpublished result) and will now be employed in a small animal heart transplantation model.

Besides the classical heart markers, the concentration of released adenosine was measured during the reperfusion period. Adenosine is largely formed from ATP catabolism under conditions of acute hypoxia [19]. Because this nucleoside is a small molecule transported effectively across membranes in the heart, it may permit (in contrast to the classical heart markers) to detect oxygenation problems at a time of preserved myocardial integrity. Potential side effects on myocardial oxygenation were indicated after storage of hearts in HTK augmented with LK 616 (100 μmol/l) or trolox.

The addition of the iron chelators/antioxidants improved vascular function but not myocardial function. This is in line with results obtained with isolated cells which showed that cardiomyocytes appear to be less susceptible to iron-dependent cold-induced injury than coronary endothelial cells (T. Noll et al., unpublished result). Although myocardial function is crucial and determines the short-term outcome of cardiac transplantation, initial endothelial/vascular injury is believed to set the stage for chronic allograft vasculopathy, a major problem in the later postoperative course [24–28]. By decreasing the initial vascular injury, the development of chronic vasculopathy should be delayed and long-term outcome of the grafts improved, which would be highly desirable clinically.

In conclusion, the present study provides evidence that preservation of vascular function is feasible even after prolonged cardioplegic arrest (8 h). Three different antioxidants or radical scavengers (LK 616, deferoxamine and trolox) improved vessel function as compared with traditional HTK. However, limitations still exist with respect to side effects of LK 616. Using animal models of cardiac transplantation future research should address the combination of LK 616 at a side effect-free concentration with deferoxamine as a protective means against the development of chronic allograft vasculopathy.

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